ENVIRONMENT, WELL-BEING, AND BEHAVIOR

Effect of Various Combinations of Alfalfa and Standard Layer Diet on Susceptibility of Laying Hens to *Salmonella* Enteritidis During Forced Molt

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ABSTRACT Feed deprivation is commonly used by the poultry industry to induce molting and stimulate multiple egg-laying cycles. However, feed deprivation has been observed experimentally to increase susceptibility of poultry to *Salmonella* infections. Previous studies indicated that alfalfa was efficacious in reducing *Salmonella*; the present investigation was designed to evaluate the efficacy of combined alfalfa and layer diets on *Salmonella* colonization. Leghorn hens over 50 wk of age were divided into 12 groups of hens and placed in individual laying cages. One week prior to dietary changes, hens were put on an 8L:16D photoperiod that continued for the 9-d experiment. Hens were challenged orally with 104 cfu of *Salmonella* Enteritidis (SE) on d 4 of treatment and cultured for SE at the termination of the 9-d study. Two independent experiments were conducted consisting of the following treatment groups: nonfed hens, full-fed standard commercial layer diet, 100% alfalfa meal diet, a 90% alfalfa meal/10% standard commercial layer diet, and a 70% alfalfa meal/30% standard commercial layer diet. When evaluating SE colonization in the ceca (Exp. 1), a reduction ($P < 0.05$) was seen in the 100% alfalfa meal diet and the 70% alfalfa meal/30% standard commercial layer diet treatment groups when compared with the controls with Log_{10} values of 0.54, 0.44, and 2.82, respectively. Evaluation of physiological parameters showed the alfalfa treatment groups had reductions ($P < 0.05$) in weight loss, ovary weight, and feed consumption when compared with the full-fed standard commercial layer diet hens, and these results were comparable with the nonfed hens. In Exp. 2, all of the treatment groups had a reduction ($P < 0.05$) in SE colonization of the ceca when compared with the controls. There were also similar physiological reductions in weight loss, ovary weight, and feed consumption when birds were fed the alfalfa diets in Exp. 2. These data suggest that alfalfa can potentially be combined with layer ration to limit SE infection and still induce a molt comparable with feed withdrawal.

Key words: molting, *Salmonella*, chicken, alfalfa, alternative diet

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INTRODUCTION

Salmonellosis is one of the most common foodborne diseases with an estimated 800,000 to 4 million human infections reported each year in the United States alone. In 2000, FoodNet published their annual report showing *Salmonella* to be the second leading cause of foodborne illness with a total of 4,237 reported cases, which was just behind *Campylobacter* with 4,640 reported cases. Of the 3,663 *Salmonella* isolates that were serotyped, the most commonly identified serotypes were Typhimurium (862 cases), Enteritidis (565), Newport (399), and Heidelberg (248; Foodnet Annual Report, 2000). Contamination of egg products has been shown to be the most commonly linked source of human foodborne illness (Patrick et al., 2004). It has been shown that the high incidence of *Salmonella enterica* serovar Enteritidis (**SE**) infection may be linked to the specific stressful management practice of inducing a molt by feed deprivation to stimulate multiple egg-laying cycles in hens (Durant et al., 1999; Holt, 2003; Ricke, 2003; Park et al., 2004). Holt (1993) showed that the mean infectious dosage ($\mathbf{ID_{50}}$) for fed hens was approximately 5.6×10^4, and birds undergoing a nonfed molt only needed 10 *Salmonella* bacteria to become infected. Feed withdrawal is the primary method used in the layer industry to induce molting but has been shown experimentally to increase SE recovery from crops, increase invasion of organs in chickens, and increase horizontal transfer in flocks (Durant et al., 1999; Holt, 2003; Ricke, 2003).

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Using diets that retain protective microflora during an induced molt would provide poultry producers with dietary approaches that could help reduce some of the enteric bacteria and physiological stresses associated with molting. Bacteria in the gastrointestinal tract derive most of their nutritional requirements for reproduction and growth from dietary components. These nutritional components are either not broken down by digestive fluids or absorbed slowly enough that bacterial populations can compete for them. Because many bacteria utilize different substrates for growth, it is important to understand that the dietary composition largely determines the microbial makeup of the gastrointestinal tract (Apajalahti and Bedford, 2000; Apajalahti, 2005; Lan et al., 2005). Specific species of bacteria can be selected for by administering certain feed ingredients that are specifically utilized by the bacteria and not by the host, including dietary fiber and oligosaccharides.

Alfalfa, with its high fiber content, has been shown to have a very long transit time in the gastrointestinal tract of chickens. This increase in transit time favors bacterial degradation of dietary fiber into fermentable substrates such as fructooligosaccharides to short chain fatty acids. Increasing the fiber content of a diet benefits the digestive system by normalizing colonic function and by increasing fecal weights and evacuation frequency (Salvin et al., 1985). These actions would help maintain the small and large intestine by increasing mucosal structure and function as well as increasing the commensal bacteria in the gastrointestinal tract (Buddington et al., 1999).

Increasing welfare concerns over feed withdrawal in molted hens has created a need to investigate alternative diets for inducing molt. There have been many natural products used as diets to induce molting including grape pomace, wheat middlings, cottonseed meal, jojoba meal, and alfalfa (McKeen, 1984; Zimmerman and Andrews, 1987; Vermaut et al., 1998; Seo et al., 2001; Davis et al., 2002; Woodward et al., 2005). Many of the diets being evaluated utilize general nutrient limitations and alteration of the mineral balance, dietary fillers, and hormones of the diets (Bell, 2003; Park et al., 2004). There have been advances in evaluating these products on different production parameters associated with laying hens. However, further evaluation of their effects on pathogens that infect these birds during a molt is needed. Recently, we have shown that combining alfalfa with layer ration still induces an effective molt and retains postmolt performance comparable with feed withdrawal (Donalson et al., 2005). Our focus in the current study is to further evaluate the addition of standard layer ration with alfalfa meal for efficacy in reducing SE from force-molted hens.

MATERIALS AND METHODS

Experimental Design

Single comb White Leghorn hens (Hy-Line International, Dallas Center, IA) over 50 wk of age were obtained from a local commercial laying flock. Laying hens were placed in wire layer cages and provided free access to water and a balanced unmedicated corn-soybean-meal-based mash layer diet that met or exceeded the National Research Council recommendations for nutrients (1994). The layer diet was calculated to provide 2,818 kcal of metabolizable energy per kg, 16.5% crude protein, 3.5% calcium, and 0.48% available phosphorus. Alfalfa is very high in crude fiber (24.1%), has a moderate protein level (17.5%), and has a low metabolizable energy value (1,200 kcal/kg; NRC, 1994). Feed samples (25 g) and fecal samples (1 g) were collected and examined for salmonellae by successive culturing in tetrathionate (Becton, Dickinson and Company, Sparks, MD) broth and brilliant green agar (**BGA**; Becton, Dickinson and Company) as described by Andrews et al., 1995). All the hens and feed used in this study tested negative for *Salmonella*. Hens were allowed to acclimate for 2 wk, followed by random assignment to 2 replicate experiments of 12 hens in each of 5 treatment groups, designated as follows: (1) Nonfed hens (**NF**); (2) Full-fed hens (**FF**); (3) alfalfa meal (**ALF**); (4) alfalfa meal 90% with 10% standard layer ration (**ALF90**); or (5) alfalfa meal 70% with 30% standard layer ration (**ALF70**). On d 4 of the molting procedure, all hens in each treatment group were challenged by crop gavage with 1 mL of 10^6 cfu of novabiocin and nalidixic acid- (**NO** and **NA**; Sigma Aldrich Co., St. Louis, MO) resistant SE. The challenge dosage is slightly higher than the 5.6×10^4 cfu dosages reported previously to be the ID_{50} for SE in nonmolted hens (Holt, 1993).

At the conclusion of the study, all hens were euthanized by cervical dislocation, and the crop, ceca, liver, spleen, and ovary were excised aseptically. Each crop was excised, aseptically opened, and the entire crop and contents together with 10 mL of sterile distilled water were blended for 1 min in a stomacher 80 lab blender (Stewart Medical, London, UK), and then serial dilutions were performed. Samples of the crop, ceca, liver, spleen, and ovary of each hen were cultured for SE.

Molt Procedure

Feed deprivation by a modification (Holt, 1993) of a previously described procedure (Brake et al., 1982) was used to induce molt. Seven days before feeding the alfalfa diets, hens were exposed to an 8L:16D photoperiod, which was continued throughout the experiment. Beginning on d 0, feed was withdrawn or hens received one of the alfalfa-based diets for 9 d, after which the study was terminated.

Bacterial Strain

A primary poultry isolate of SE (phage type 13A) from the National Veterinary Services Laboratory (Ames, IA), selected for resistance to NO and NA in the USDA-ARS facility (College Station, TX) was used. Media to culture the resistant isolate in experimental studies contained 25 μg of NO and 20 μg of NA per mL. The challenge inoculum was prepared from an overnight culture, which had

Table 1. Effects of alfalfa on incidence and colonization of *Salmonella* Enteritidis (SE) in force-molted hens, Experiment 1

	Log_{10} of SE/g of contents[2]		SE culture positive hens		
Treatment[1]	Ceca	Crop	Liver	Spleen	Ovary
NF	2.82 ± 0.59[a]	0.99 ± 0.24[a]	7/12	4/12	5/12
FF	0.00 ± 0.04[b]	0.00 ± 0.00[b]	0/12*	0/12*	1/12
ALF	0.54 ± 0.44[b]	0.49 ± 0.23[ab]	0/12*	1/12	0/12*
ALF90	1.46 ± 0.44[ab]	0.00 ± 0.02[b]	2/12*	2/12	0/12*
ALF70	0.44 ± 0.13[b]	0.28 ± 0.10[ab]	2/12*	0/12*	1/12

[a,b]Mean values within the same column with no common superscripts differ significantly ($P \leq 0.05$).

*A significant difference was found between the number of positive controls and positive treated crops or ceca ($P \leq 0.05$).

[1]NF = nonfed; FF = full fed; ALF = alfalfa diet; ALF90 = alfalfa 90% + 10% corn soy; ALF70 = alfalfa 70% + 30% corn soy.

[2]Values represent the mean of 12 hens per treatment.

been previously transferred 3 times in trypticase soy broth (Becton, Dickinson and Company). The culture was serially diluted in sterile phosphate-buffered saline to a concentration of approximately 10^6 cfu per mL. The cfu of the challenge inoculum was confirmed by plating onto BGA plates.

Recovery of Salmonella

All samples tested for *Salmonella* (+/–) including ceca, crop, liver, spleen, and ovary were minced with sterile scissors and cultured. The organ samples were incubated for 24 h at 41°C in Rappaport-Vassiliadis R10 broth (Becton, Dickinson and Company). After incubation, the broth was streaked onto a BGA plate containing 25 μg of NO/mL and 20 μg of NA/mL, incubated for an additional 24 h at 37°C, and examined for the presence of SE colonies. Samples that were direct plated for total cfu were stomached, and 0.25 g of cecal or crop contents was placed into a 6-mL snap cap polypropylene tube containing 2.25 mL of Butterfield's solution. Serial dilutions of each sample were performed using 0.5 mL of the sample, placed into 4.5 mL of Butterfield's solution for a final concentration of 10×, 100×, and 1,000×. One hundred microliters from each dilution tube was placed onto a BGA plate and spread plated using a bacterial cell spreader. All of the plates were incubated for 24 h at 37°C, and the number of *Salmonella* cfu were determined and expressed as $\log_{10}$ *Salmonella* per gram of cecal or crop contents. Cecal and crop contents that were negative at a 100-fold dilution on BGA plates but were positive at a 10-fold dilution on BGA plating were assigned 1.00 $\log_{10}$ *Salmonella* per gram of cecal contents (Corrier et al., 1993; 1995). Suspect colonies were confirmed by biochemical tests on triple sugar-iron agar and lysine-iron agar (Oxoid, Unipath Ltd., Hampshire, UK) and further identified as SE serologically using *Salmonella* O antiserum group D, factors 1, 9, and 12.

Statistical Analysis

Chi-square analysis was used to determine significant differences between treatment groups for incidences of SE colonization of the crop, ceca, liver, spleen, and ovary (Luginbuke and Schlotzhauer, 1987). Differences in the Log_{10} cfu of SE counts among treatment groups were determined by ANOVA using the GLM procedures. Significant differences were further separated using Duncan's multiple range tests and commercial statistical analysis software (SAS Institute, Cary, NC; Luginbuke and Schlotzhauer, 1987). All data analyzed by statistical analyses were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

In the present investigation, we evaluated the effects of feeding different concentrations of a standard laying ration mixed with an alfalfa-based diet. In Experiment 1, there was a significant reduction ($P < 0.05$) of SE; the Log_{10} values of the ceca were reduced from 2.82 in the NF controls to 0.54 and 0.44 in the ALF and ALF70 treatment groups, respectively. When evaluating the crops, there was also a significant reduction in the Log_{10} values of SE; the NF controls had 0.99 cfu and were reduced to 0.0 in the ALF90 treatment group. *Salmonella* is known to be an invasive bacterium that infects many internal organs. Microbial cultures from the internal organs showed that all 3 treatments significantly reduced ($P < 0.05$) the incidence of *Salmonella* from the liver with 7/12 (58%) infectivity in the NF controls compared with 0/12 (0.0%), 2/12 (17%), and 2/12 (17%) in the ALF, ALF90, and ALF70 treatment groups, respectively (Table 1). Microbial cultures of the spleen also showed a significant reduction in the ALF70 treatment group, and SE was also significantly reduced in the ovaries when the birds were fed ALF or ALF90. In Experiment 2, similar results were observed in reductions ($P < 0.05$) of SE when evaluating the ceca and the crop. The SE was reduced in the ceca of all 3 experimental groups ALF (1.99 Log_{10}), ALF90 (1.32 Log_{10}), and ALF70 (0.25 Log_{10}) when compared with the NF control (4.68 Log_{10}). In the crop, reductions ($P < 0.05$) of SE were also seen with mean values of 0.25 and 0.08 in the ALF90 and ALF70 treatment groups, respectively, compared with NF controls at 1.22 Log_{10}. Microbial cultures from the internal organs in Experiment 2 showed significant reductions in the liver, spleen, and ovaries of birds fed the ALF90 and ALF70 diets (Table 2).

Table 2. Effects of alfalfa on incidence and colonization of *Salmonella* Enteritidis (SE) in force-molted hens, Experiment 2

Treatment[1]	Log_{10} of SE/g of contents[2]		SE culture positive hens		
	Ceca	Crop	Liver	Spleen	Ovary
NF	4.68 ± 0.68[a]	1.22 ± 0.26[a]	11/12	7/12	6/12
FF	0.09 ± 0.09[c]	0.36 ± 0.15[b]	3/12**	0/12*	2/12
ALF	2.99 ± 0.66[b]	1.06 ± 0.32[a]	8/12	4/12	5/12
ALF90	1.32 ± 0.64[c]	0.25 ± 0.13[b]	5/12*	2/12*	1/12*
ALF70	0.25 ± 0.13[c]	0.08 ± 0.08[b]	2/12**	1/12*	0/12*

[a–c]Mean values within the same column with no common superscripts differ significantly ($P \leq 0.05$).

*,**A significant difference was found between the number of positive controls and positive, treated crops, or ceca (*$P \leq 0.05$, **$P \leq 0.001$).

[1]NF = nonfed; FF = full fed; ALF = alfalfa diet; ALF90 = alfalfa 90% + 10% corn soy; ALF70 = alfalfa 70% + 30% corn soy.

[2]Values represent the mean of 12 hens per treatment.

Currently, the commercial poultry industry utilizes feed withdrawal as the primary means to induce molting and stimulate multiple egg-laying cycles in layer hens (Brake, 1993; Holt, 1995). The stress of this commercial practice has been shown to increase the susceptibility of hens to SE, which can be seen by increased intestinal shedding and internal organ invasion (Holt and Porter, 1992; Holt et al., 1995; Thiagarajan et al., 1994). The primary mechanism for egg contamination has been linked to the invasion of this pathogen into the reproductive organs after initial infection (Gast and Beard, 1990; Shivaprasad et al., 1990). In the present investigation, the use of alternative molting diets significantly reduced the overall numbers and incidence of SE in the crop, ceca, and internal organs of SE-challenged birds. Reducing the incidence of this bacterium in the internal organs of birds could reduce the possibility of contamination in breeder or table egg operations. It has been shown that *Salmonella* infections can be transmitted vertically to subsequent flocks that can maintain this contamination for long periods (Humphrey, 1999). Reducing the overall incidence of this pathogen will decrease the transovarian transmission of this bacterium into commercial table egg operations. Although the use of alternative molting diets does not completely eliminate *Salmonella* infection, these diets can significantly reduce pathogens. Alternative molting diets with minor alterations and manipulations could even further reduce the overall numbers and incidence levels of SE in molted hens.

When evaluating physiological parameters in Experiment 1, all the alfalfa treatment groups had reductions ($P < 0.05$) in weight loss, ovary weights, and feed consumption when compared with the FF hens, and these results were comparable with the NF hens (Table 3). Birds in the NF group had a reduction of 34.7% in body weight and 30.2, 26.4, and 21.6% reductions in the ALF, ALF90, ALF70 treatment groups, respectively, compared with the FF treatment group that had a 2% reduction. Similar results were seen in Experiment 2 with all the treatment groups having reductions ($P < 0.05$) in body weight (Table 4). Significantly reducing the ovary weight is another physiological parameter that is indicative of a successful molting procedure. In the present investigation all treatment groups in Experiment 1 were significantly reduced ($P < 0.05$) compared with the FF treatment group. The postmolt ovary weight of the FF group was 43.14 g compared with 5.66, 5.93, 6.04, and 6.29 g weights in the NF, ALF, ALF90, ALF70 treatment groups, respectively. Similar results were seen in Experiment 2 with all treatment groups having reductions ($P < 0.05$) in postmolt ovary weights. The ALF70 showed the largest amount of feed consumption (g/bird/d) with 36.1 g, which was significantly higher than ALF and ALF90 with 6.8 and 18.1 g, respectively.

Induced molting is a procedure used by the commercial industry to increase egg quality and productivity for multiple egg-laying cycles. There have been many methodologies used to transition birds into a molting period, which

Table 3. Evaluation of weight loss, ovary weight, and feed consumption in Experiment 1

Treatment[1]	Total body weight (g) loss (%)	Ovary weight (g) postmolt	Feed consumption (g/bird per d)
NF[2]	439.7 ± 21.7[a] (34.7)	5.66 ± 0.35[b]	—
FF	31.2 ± 9.7[d] (1.8)	43.1 ± 2.5[a]	111.8[a]
ALF	492.8 ± 15.8[ab] (30.2)	5.93 ± 0.46[b]	6.8[d]
ALF90	354.4 ± 12.8[bc] (26.4)	6.04 ± 0.54[b]	18.1[c]
ALF70	308.9 ± 15.7[c] (21.6)	6.29 ± 1.4[b]	36.1[b]

[a–d]Mean values within the same column with no common superscripts differ significantly ($P \leq 0.05$).

[1]NF = nonfed; FF = full fed; ALF = alfalfa diet; ALF90 = alfalfa 90% + 10% corn soy; ALF70 = alfalfa 70% + 30% corn soy.

[2]Values represent the mean of 12 hens per treatment.

Table 4. Evaluation of weight loss, ovary weight, and feed consumption in Experiment 2

Treatment[1]	Total body weight (g) loss (%)	Ovary weight (g) postmolt	Feed consumption (g/bird per d)
NF[2]	466.4 ± 14.0[a] (35.3)	7.42 ± 0.44[c]	—
FF	47.4 ± 14.0[d] (2.8)	40.18 ± 2.0[a]	99.1[a]
ALF	445.9 ± 21.8[ab] (33.1)	7.82 ± 0.63[cb]	7.2[c]
ALF90	400.5 ± 15.6[b] (27.7)	8.18 ± 0.55[cb]	18.1[c]
ALF70	341.2 ± 21.8[c] (23.4)	12.37 ± 2.6[b]	33.8[b]

[a–d]Mean values within the same column with no common superscripts differ significantly ($P \leq 0.05$).

[1]NF = nonfed; FF = full fed; ALF = alfalfa diet; ALF90 = alfalfa 90% + 10% corn soy; ALF70 = alfalfa 70% + 30% corn soy.

[2]Values represent the mean of 12 hens per treatment.

include feed or nutrient restriction, dietary meals, artificial drugs, and hormones or hormone agonists. Webster (2003) describes the 3 phases involved in fasting. Phase 1, the adaptation phase, is very brief, only lasting a few days. During this phase there is a rapid reduction in body mass as well as a reduction in metabolic rate. Plasma corticosterone levels increase due to the stress of feed withdrawal. However, it has been shown that immobilization or crating of chickens causes higher levels of plasma corticosterone than feed withdrawal, possibly indicating lower levels of stress in molted hens (Beuving and Vonder, 1978). Birds in phase 1 quickly deplete their hepatic glycogen stores, and gluconeogenesis is established. In phase 2, daily body mass loss levels off and remains somewhat constant, with slight declines with extended duration. Birds' metabolic activity remains constant during this phase. To conserve energy, birds' movements are restricted; almost all energy expenditure is a result of fat catabolism. It is not until phase 3 that protein catabolism is initiated. Birds in the commercial setting never reach phase 3. Further evaluations are needed to completely understand the physiological and metabolic effects of alfalfa as an alternative molting diet. It is important to make sure that birds advance through these stages in normal time periods to ensure that phase-based stresses are minimized. Molting programs are used to cease egg production and enter the bird into a nonreproductive state; these programs typically last for 10 to 14 d, and body weight losses are typically 25 to 35% (UEP, 2002; Bell, 2003; Webster, 2003). The weight reductions in the present investigations are comparable with the recommended weight losses during molting. In both experiments presented here, the alfalfa treatment groups had comparable ovary weight (g) postmolt with the NF treatment group. These alfalfa-based diets did show good results in reducing the measured physiological parameters associated with molting.

In the present investigation, feeding an alfalfa diet or an alfalfa diet supplemented layer ration diet was effective in reducing the incidence and severity of colonization and organ invasion by SE. When evaluating physiological parameters, the alfalfa treatment groups showed reductions in body weight and ovary weights that were comparable with NF birds. Using these combinations of alfalfa would allow the industry an opportunity to possibly reduce stress factors associated with feed deprivation and bacterial pathogen loads from commercial poultry. Utilizing these new innovative technologies could potentially reduce the incidence or numbers of these foodborne pathogens from entering the food chain.

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